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Tumor necrosis factor-α induces adhesion molecule expression through the sphingosine kinase pathway

PU XIA*, JENNIFER R. GAMBLE*†, KERRY-ANNE RYE‡, LIJUN WANG*, CHARLES S. T. HII§, PETER COCKERILL*, YEESIM KHEW-GOODALL*, ANDREW G. BERT*, PHILIP J. BARTER‡, AND MATHEW A. VADAS*†¶

*Division of Human Immunology, †Department of Lipid Research, The Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science and University of Adelaide, and §Department of Immunology, Women's and Children's Hospital, Adelaide, SA 5000, Australia

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ABSTRACT **The signaling pathways that couple tumor necrosis factor-α (TNFα) receptors to functional, especially inflammatory, responses have remained elusive. We report here that TNFα induces endothelial cell activation, as measured by the expression of adhesion protein E-selectin and vascular adhesion molecule-1, through the sphingosine kinase (SKase) signaling pathway. Treatment of human umbilical vein endothelial cells with TNFα resulted in a rapid SKase activation and sphingosine 1-phosphate (S1P) generation. S1P, but not ceramide or sphingosine, was a potent dose-dependent stimulator of adhesion protein expression. S1P was able to mimic the effect of TNFα on endothelial cells leading to extracellular signal-regulated kinases and NF-κB activation, whereas ceramide or sphingosine was not. Furthermore, *N,N*-dimethylsphingosine, an inhibitor of SKase, profoundly inhibited TNFα-induced extracellular signal-regulated kinases and NF-κB activation and adhesion protein expression. Thus we demonstrate that the SKase pathway through the generation of S1P is critically involved in mediating TNFα-induced endothelial cell activation.**

Tumor necrosis factor-α (TNFα) was originally described for its antitumor activity, but is now recognized to be one of the most pleiotropic cytokines in mediating systemic inflammatory and immune responses (1, 2). A major site for these TNFα actions is the vascular endothelium, where TNFα triggers endothelial cells to secrete various cytokines and induces or enhances the expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 and E-selectin (3). The regulated expression of these adhesion molecules is essential for the recruitment of circulating blood cells to the endothelium during the inflammatory and immune responses (3–5).

TNFα activity is exerted through binding two distinct membrane receptors, p55 (TNFα-R1) and p75 (TNFα-R2). Engagement of the TNFα receptors results in recruitment of two distinct classes of receptor-associated proteins, one the TRADD, FADD/MORT1 and RIP family, and the other, the TRAF family (6–8). Both of these appear to couple TNFα receptors to downstream signaling cascades such as cysteine proteases and NF-κB activation to regulate cell proliferation, differentiation, and programmed cell death (6). Recently, the lipid second messenger, ceramide, has also received attention in TNFα signaling (6, 9). TNFα stimulates the activation of sphingomyelinase, yielding ceramide that, in turn, can induce apoptosis and may play a role in apoptotic signaling in various cell types (6, 9). In addition, ceramide can be subsequently metabolized to sphingosine and sphingosine 1-phosphate (S1P), via ceramidase and sphingosine kinase (SKase) activation, respectively (10). These sphingomyelin metabolites were also proposed to play a variety of roles in regulation of cellular activities such as calcium mobilization, cell motility, and mitogenesis (9, 10). In this study, we demonstrate that TNFα promoted generation of ceramide that was unable to mediate the TNFα proinflammatory action in human umbilical vein endothelial cells (HUVEC). By contrast S1P was a potent inducer of adhesion molecule expression. We show that TNFα stimulated SKase activity and S1P generation in HUVEC, and blockage of SKase by its inhibitor markedly reduced the TNFα-dependent extracellular signal-regulated kinase (ERK) and NF-κB activation and adhesion molecule expression. Thus, we show that SKase activation is an important signaling event in the TNFα-induced endothelial activation, a major effect of TNFα *in vivo*.

EXPERIMENTAL PROCEDURES

Cell Culture and Flow Cytometry Analysis. HUVEC were isolated as described (11). The cells were cultured on gelatin-coated culture flasks in medium M199 with Earle's salts supplemented with 20% fetal calf serum, 25 μg/ml endothelial growth supplement (Collaborative Research), and 25 μg/ml heparin. The cells were used between passages 2 and 6 for all experiments. To measure the adhesion molecule expression, cells were plated in 24-well dishes and reached confluency. After the indicated treatment, cells were washed with medium M199 and incubated with primary monoclonal antibodies to VCAM-1, E-selectin, or an isotype-matched nonrelevant antibody for 30 min. These antibodies were generated in our laboratory and their characteristics were described previously (12). Cells were then incubated with fluorescein isothiocyanate-conjugated secondary antibody and fixed in 2.5% formaldehyde. The expression of cell-surface adhesion molecules was measured as fluorescence intensity by use of a Coulter Epics Profile XL flow cytometer.

Metabolic Labeling, Sphingolipids, and SKase Assay. To measure sphingomyelin and S1P levels, the HUVEC were labeled with [^{3}H]serine (5 μCi/ml; 1 Ci = 37 GBq) for 48 h. After TNFα stimulation for the indicated times, cellular lipids were extracted and resolved by TLC with two different solvent systems: (*i*) chloroform/methanol/acetic acid/water (50:30:8:5, vol/vol) and (*ii*) 1-butanol/acetic acid/water (3:1:1, vol/vol). Sphingolipid spots were visualized by fluorography, quantified by scintillation spectrometry, and normalized by radioactivity recovered in total cellular lipids.

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Abbreviations: DMS, *N,N*-dimethylsphingosine; ERK, extracellular signal-regulated kinase; HUVEC, human umbilical vein endothelial cells; SKase, sphingosine kinase; S1P, sphingosine 1-phosphate; TNFα, tumor necrosis factor-α; VCAM-1, vascular adhesion molecule-1; JNK, c-JUN N-terminal kinase.

†J.R.G. and M.A.V. contributed equally to this work.

¶To whom reprint requests should be addressed. e-mail: Mathew.Vadas@imvs.sa.gov.au.

Cellular ceramide was extracted and quantified with the diacylglycerol kinase reaction (13). To exclude a possible error caused by some factors in the extracts affecting diacylglycerol kinase (14), synthetic C_2-ceramide was added in assays as an internal control. There were no changes in the phosphorylated C_2-ceramide in this assay system.

SKase activity was measured *in vitro* by incubating the cytosolic fraction with 10 μM sphingosine-BSA complex and [γ-^{32}P]ATP (1 mM, 0.5 mCi/ml) for 15 min at 37°C as described previously (15). For kinetic study, cell extract was prepared from the HUVEC treated with TNFα for 5 min. The kinase assay was performed with various concentrations of sphingosine (1.25, 2.5, 5, and 10 μM) in the absence or presence of *N,N*-dimethylsphingosine (DMS) at 5 or 10 μM, respectively. The K_m value (16.4 μM sphingosine) was obtained from a double-reciprocal plot. The formation of S1P *in vivo* was measured in the permeabilized cells as described previously (15).

ERK and c-JUN N-Terminal Kinase (JNK) Activity Assays. The cells were treated with the indicated agents for 30 min, ERK activities were then assayed with myelin basic protein (MBP) as substrate after immunoprecipitation with antibodies against p42/p44^{ERK} (Santa Cruz Biotechnology). The kinase reaction products were separated on SDS/10% PAGE. In parallel, an aliquot of the same cell lysates was blotted with anti- p42/p44^{ERK} antibodies to ensure equal ERK expression. JNK activity was measured by a solid-phase assay using glutathione *S*-transferase-jun-(1–79) fusion protein as substrate as described (16).

Electrophoretic Mobility-Shift Assay. Nuclear extracts were prepared from HUVEC treated for 30 min with vehicle or the indicated agents. The double-stranded oligonucleotides used as a probe in these experiments included 5′-GGATGCCATTGGGGATTTCCTCTTTACTGGATGT-3′, which contains a consensus NF-κB binding site in the E-selectin promoter that is underlined (17). Gel mobility shift of a consensus NF-κB oligonucleotide was performed by incubating a ^{32}P-labeled NF-κB probe with 4 μg of nuclear proteins. The specific DNA–protein complexes were abolished by addition of a 50-fold molar excess of unlabeled E-selectin NF-κB oligonucleotides. The specificity of NF-κB binding complex was further identified by supershift analyses. Anti-p50 and anti-p65 polyclonal antibodies (Santa Cruz Biotechnology) were added before addition of radiolabeled NF-κB probe.

RESULTS

TNFα Stimulates Sphingomyelin Turnover in HUVEC. As ceramide has been implicated in TNFα signaling (9), we examined the effect of TNFα on the hydrolysis of sphingomyelin to ceramide in HUVEC. TNFα stimulation of HUVEC rapidly reduced sphingomyelin content to 40% of control within 30 min, with return to near basal levels by 2 h (Fig. 1). In parallel, the cellular ceramide levels were rapidly increased ($\approx$2-fold), peaking at 30 min after TNFα treatment (Fig. 1*B*). Our data indicate that TNFα induces sphingomyelin turnover and ceramide generation in HUVEC.

Ceramide Does Not Mediate TNFα-Induced Adhesion Molecule Expression. To clarify whether sphingomyelin turnover is involved in TNFα-induced adhesion molecule expression, we determined the effect of ceramide in HUVEC by using either a cell-permeant form of ceramide (C_2-ceramide) or sphingomyelinase, which generates endogenous ceramide. As shown in Fig. 2, C_2-ceramide was a poor stimulator of E-selectin and VCAM-1 expression, reaching levels that were $<$10% of that stimulated by TNFα. In parallel, the cells treated with sphingomyelinase or D-*erythro*-(*N*-myristoylamine)-1-phenyl-1-propanol, a ceramidase inhibitor (18) that induced endogenous ceramide accumulation by 3-fold (data not shown), failed to stimulate the expression of E-selectin or VCAM-1 (Fig. 2*B*). Thus, ceramide is unlikely to be a second messenger in mediating TNFα-induced adhesion protein expression.

S1P Is a Mediator in TNFα-Induced Adhesion Molecule Expression. Because not only ceramide but its metabolites, especially S1P, have been proposed to serve as modulators or signaling molecules in a variety of cellular activities, we next explored the role of S1P in endothelial cells. In contrast to ceramide, S1P profoundly stimulated cell-surface expression of E-selectin and VCAM-1 and their mRNA levels in HUVEC (Fig. 2). Data showed that S1P was a potent and dose-dependent inducer of E-selectin and VCAM-1, with an EC_{50} at $\approx$1 μM and reaching levels at 5 μM that were approximately

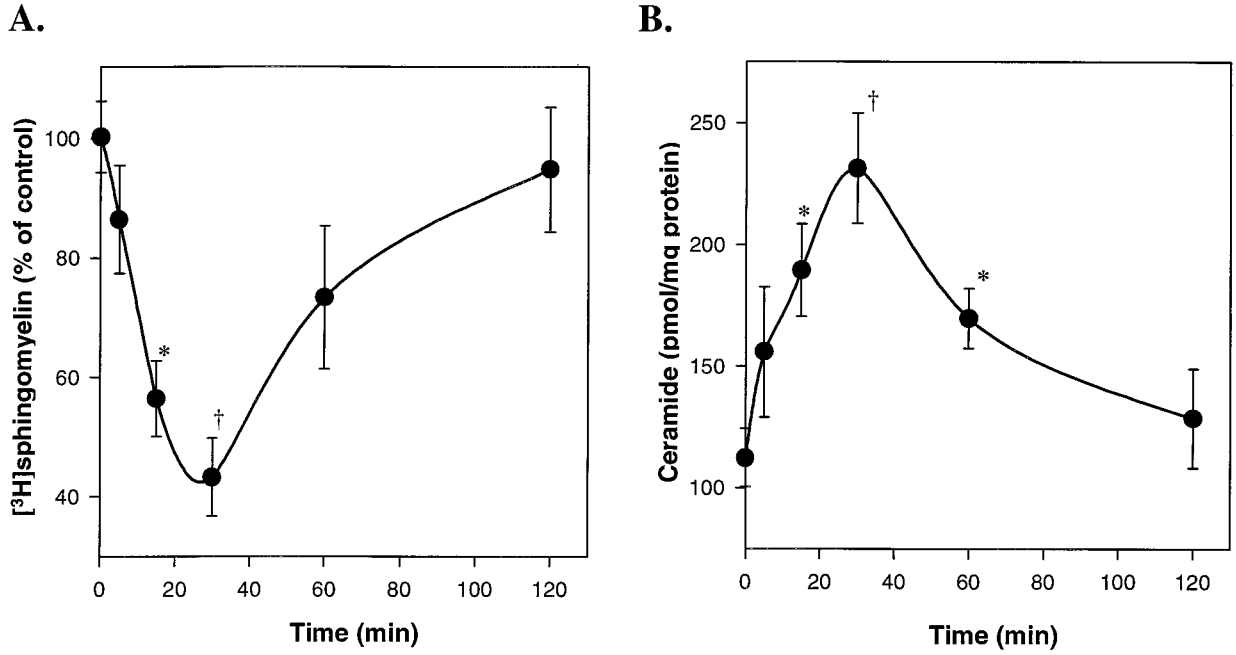

FIG. 1. TNFα-induced sphingomyelin turnover in HUVEC. (*A*) HUVEC were labeled with [^{3}H]serine for 48 h and treated with TNFα (100 units/ml), total cellular lipids were extracted, and [^{3}H]sphingomyelin was then resolved by TLC at the desired time point. (*B*) The unlabeled cells were treated with TNFα as indicated above, and cells were lysed to measure ceramide levels by using the diacylglycerol kinase assay. The results represent mean values ± SD from three independent experiments. *, $P < 0.01$; †, $P < 0.001$, vs. the basal levels.

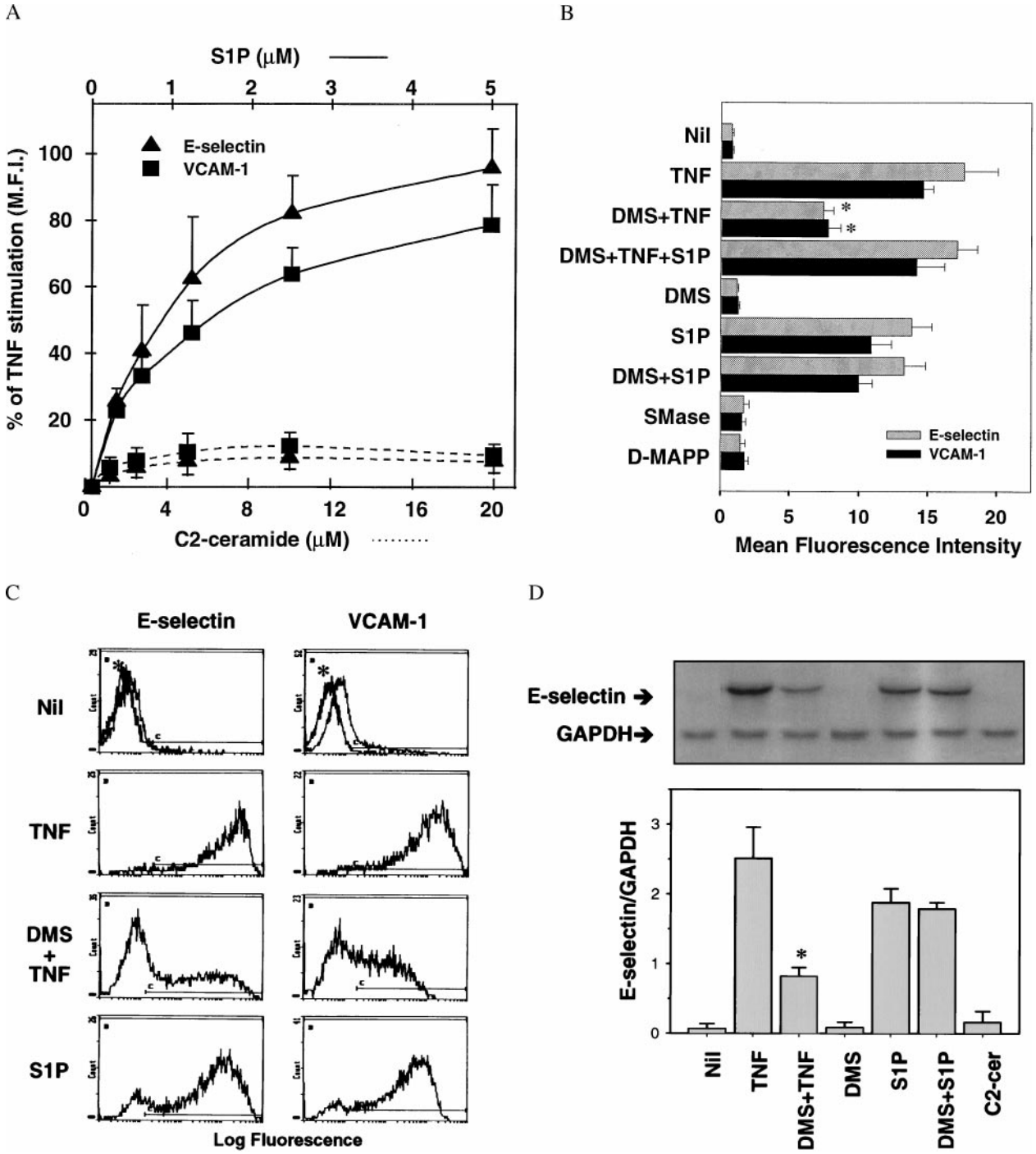

FIG. 2. Effects of C_2-ceramide and S1P on adhesion molecules expression. (*A*) HUVEC were treated with an increasing concentration of C_2-ceramide or S1P for 4 h. The cell-surface expression of E-selectin or VCAM-1 was measured by flow cytometry. The data are expressed as percent of TNFα (100 units/ml)-stimulation in the mean fluorescence intensity (M.F.I.). (*B*) The endothelial cells were treated with vehicle (Nil), S1P (5 μM), C_2-ceramide (10 μM), DMS (5 μM), sphingomyelinase (SMase, 1 unit/ml), D-*erythro*-(*N*-myristoylamine)-1-phenyl-1-propanol (D-MAPP, 5 μM), and/or TNFα (TNF, 100 units/ml), respectively, for 4 h, then the cell-surface E-selectin or VCAM-1 was measured. (*C*) Flow cytometry profiles showed the effect of S1P on expression of E-selectin (*Left*) and VCAM-1 (*Right*). Asterisks indicate a negative control profile with the isotype-matched nonrelevant antibody. (*D*) After the indicated treatment for 4 h, E-selectin mRNA levels were measured by Northern blot assay with α-^{32}P-labeled cDNA probes (12). Bar graph (*Bottom*) depicts relative levels of E-selectin mRNA quantified by the PhosphorImager and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Values in *A*, *B,* and *D* represent mean ± SD from at least three independent experiments. *, $P < 0.01$, compared with TNFα stimulation.

equivalent to 100 units/ml TNFα (Fig. 2 *A–C*). As a control, sphingosine had no effect on the expression of adhesion molecules (data not shown), suggesting a specific effect of S1P in HUVEC. To further examine the role of S1P in TNFα-mediated activation, the formation of intracellular S1P was inhibited by a competitive inhibitor of SKase, DMS. DMS markedly decreased the TNFα-induced adhesion protein expression and mRNA levels by 50–70% (Fig. 2 *B–D*), indicating that SKase activation is an important signaling event in the TNFα action. Under the same conditions, DMS had no cytotoxic effect on HUVEC measured by cell viability (MTT assay) and morphological analysis (data not shown). Con-

versely, the DMS-inhibited TNFα action was reversed by the addition of S1P, and DMS did not prevent S1P-induced adhesion protein expression (Fig. 2*B*), further suggesting the linkage between S1P and TNFα signaling in the endothelial cell activation.

TNFα Induces SKase Activation and S1P Generation in HUVEC. Given the potent stimulatory role of S1P for the signaling in TNFα-induced adhesion protein expression, we sought to determine the effect of TNFα on S1P generation. As S1P is generated from sphingosine by SKase (19), we first measured SKase activity both *in vitro* and *in vivo*. TNFα stimulation of HUVEC caused a rapid and transient increase in cytosolic SKase activity, reaching a maximum of 165% ± 13% ($P < 0.01$) of basal within 5 min (Fig. 3*A*). Kinetic study of the SKase indicated that it followed Michaelis–Menten kinetics. The K_m value of activation was 16.4 μM sphingosine, obtained from *in vitro* determination performed with various concentrations of sphingosine in the presence of an increasing concentration of DMS (Fig. 3*A*, *Inset*). Fig. 3*B* shows that TNFα induced a time-dependent increase in the formation of S1P *in vivo*, which was in parallel with the SKase activity *in vitro*. To confirm the effect of TNFα on SKase activation, the levels of S1P in intact cells measured from the [^{3}H]serine-labeled HUVEC clearly demonstrated the activation of SKase by TNFα stimulation (Fig. 3*B, Inset*). Addition of DMS at a concentration of 5 μM completely normalized the TNFα-induced increase in S1P levels, indicating a specificity of inhibition on SKase activity in HUVEC.

SKase Activation Is Involved in TNFα-Induced Activation of ERK and NF-κB. Members of the mitogen-activated protein kinase family are important mediators of signals transduced from the cell surface to intracellular responses and gene expression. The ERK, an archetypal member of this family, has been proposed to mediate endothelial activation by a variety of stimuli (20). We thus examined the role of SKase in ERK activation. Fig. 4*A* shows that both TNFα and S1P were approximately equipotent in stimulating ERK activities, whereas C_2-ceramide did not (data not shown). Treatment with DMS inhibited TNFα-activated ERK by 51% ± 14% ($P < 0.02$), showing a role for SKase in the TNFα-activated ERK signal cascade. Fig. 4*B* shows that S1P failed to activate JNK and DMS did not interrupt TNFα-induced JNK activation, suggesting that JNK may not be involved in SKase pathway.

Because the transcription factor NF-κB is essential for regulation of TNFα-induced adhesion protein gene transcription (21), the role of activated SKase pathway in activation of NF-κB was investigated. Electrophoretic mobility-shift assay showed that treatment of cells with DMS markedly inhibited the TNFα-induced activation of NF-κB by 62% ± 16% ($P < 0.01$, Fig. 5 *A* and *D*), but did not inhibit other transcription factors such as Oct-1 activation (Fig. 5*B*), indicating the specificity of SKase role in TNFα-promoted NF-κB activation. In confirming the effect of SKase activity in NF-κB activation, Fig. 5*C* shows that treatment of HUVEC with S1P induced a significant nuclear NF-κB accumulation. The composition of S1P-induced NF-κB specific protein–DNA complexes were identical to that induced by TNFα, which was revealed to be p50/p65 heterodimer by antibody supershift assay and by competition analyses. Taken together, these results indicate that the activation of SKase is necessary in human endothelial cells for TNFα-stimulated NF-κB activation, a critical component in TNFα-induced adhesion protein expression.

DISCUSSION

The expression of adhesion proteins on activated endothelial cells plays a major role in the recruitment of blood cells to the endothelium during the inflammatory responses (3–5). The nature of the inflammatory signals and associated molecular mechanisms that activate adhesion molecule expression in endothelial cells are unknown. Factors commonly found in inflammatory lesions, such as TNFα and interleukin 1, induce the expression of adhesion molecules in cultured endothelial cells. Thus, TNFα-stimulated adhesion molecule expression on HUVEC provided a useful model to investigate the signal pathway in the regulation of endothelial cell activation. Using

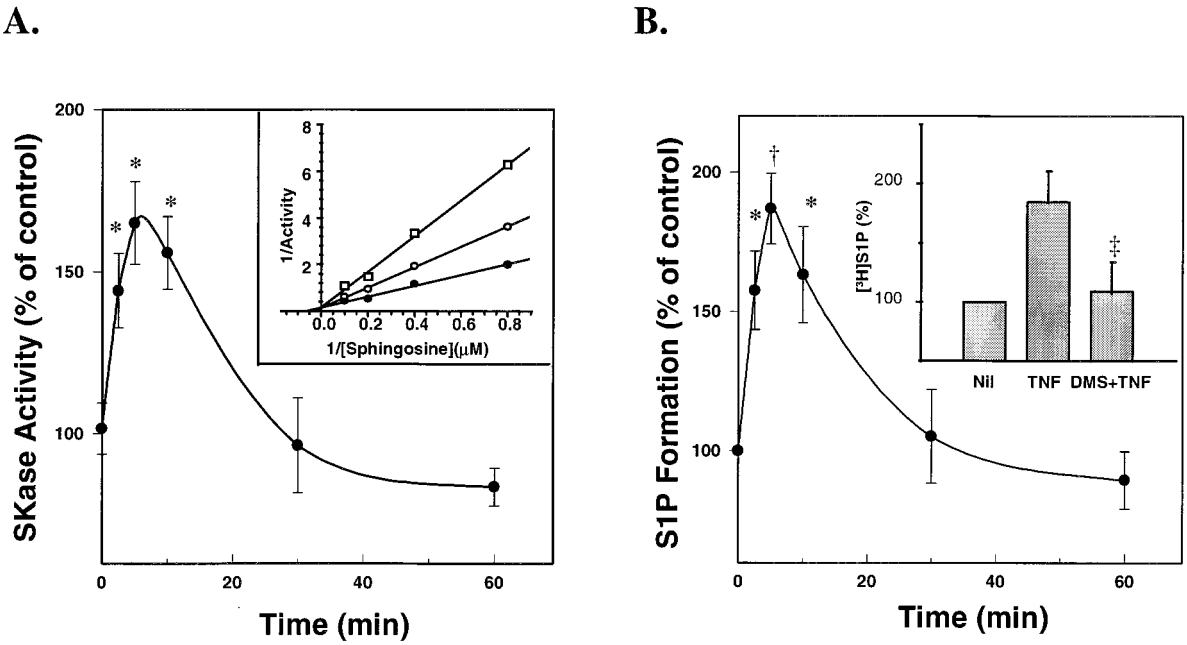

FIG. 3. TNFα-induced SKase activation. (*A*) HUVEC treated with TNFα (TNF, 100 units/ml) at the desired time points, the cytosolic fractions were extracted to measure SKase activity. (*Inset*) Kinetic study of SKase. The cell extract was prepared from the cells treated with 100 units/ml TNFα for 5 min. The kinase assay was performed with various concentrations of sphingosine in the absence (●) or presence of 5 μM (○) or 10 μM (□) DMS. (*B*) After treatment with TNFα as described above, the cells were permeabilized to measure the production of S1P *in vivo*. (*Inset*) S1P levels in intact cells measured by labeling with [^{3}H]serine. The data in *A* and *B* are mean values ± SD of three individual experiments. *, $P < 0.01$; †, $P < 0.001$, vs. the basal levels; ‡, $P < 0.001$, vs. TNFα stimulation.

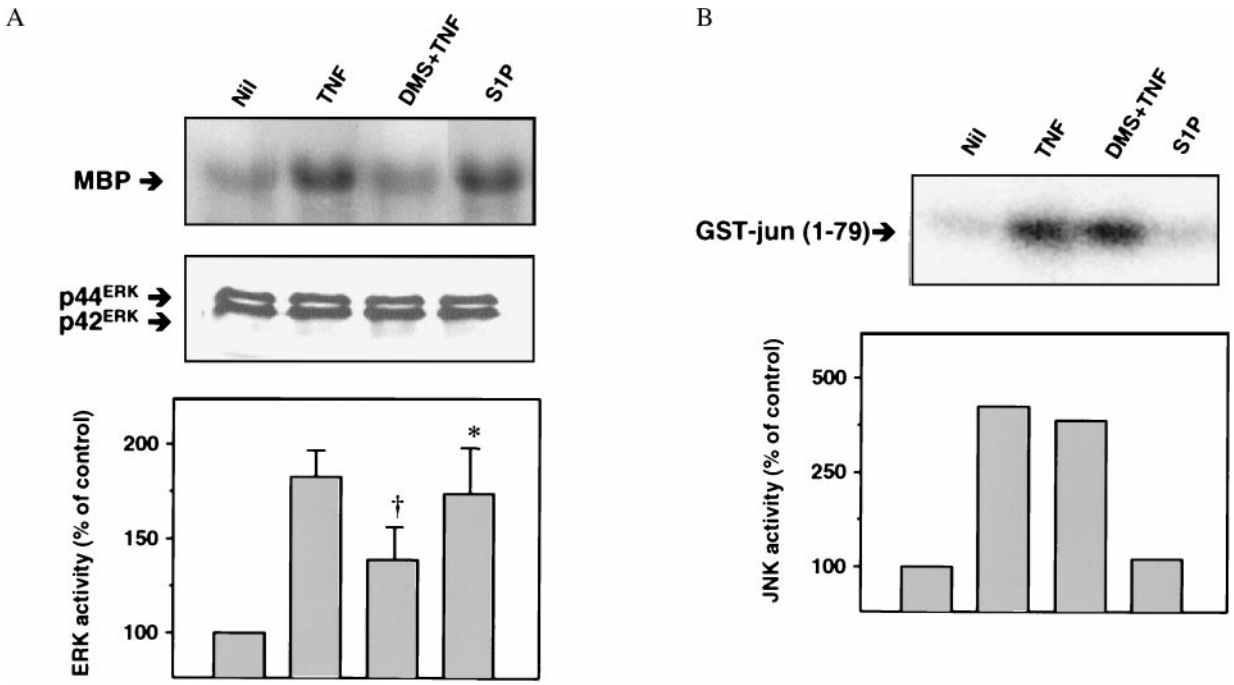

FIG. 4. Effect of SKase activity on ERK and JNK activation. (*A*) The cells were treated with the indicated agents for 30 min, and ERK activities were then assayed with myelin basic protein (MBP) as substrate after immunoprecipitation with antibodies against p42/p44^{ERK}. The kinase reaction products were separated on SDS/10% PAGE. In parallel, an aliquot of the same cell lysates was blotted with anti-p42/p44^{ERK} antibodies to ensure equal ERK expression. Bar graph (*Bottom*) depicts ERK activity quantified by the PhosphorImager. Values represent mean ± SD in percent of control from three independent experiments. *, $P < 0.01$, compared with control; †, $P < 0.02$, vs. TNFα (TNF) stimulation. (*B*) JNK activity was measured with glutathione *S*-transferase-jun-(1–79) as substrate after the treatment as described above. Bar graph (*Bottom*) shows JNK activity quantified by the radioactivity incorporated into glutathione *S*-transferase (GST)-jun-(1–79). Data represent mean of two separate experiments.

this experimental model, we demonstrated that SKase pathway is critically involved in TNFα proinflammatory action.

Ceramide has been suggested to couple the TNFα receptors to certain downstream events by activated sphingomyelinase (9, 22). However, the role of ceramide has been controversial. Indeed, we showed that whereas TNFα induced sphingomyelin turnover and ceramide generation in human endothelial cells (Fig. 1), ceramide failed to mimic the effect of TNFα on stimulation of adhesion molecule expression. This finding concurs with other observations that questioned the physiological significance of ceramide, with the evidence suggesting its effect unlikely to be relevant (9, 23, 24). In fact, we have shown that ceramide inhibits the expression of adhesion molecules by TNFα stimulation (unpublished results). Thus, it is unlikely that ceramide can serve as a second messenger to couple TNFα receptors and adhesion molecules expression in endothelial cells.

S1P is now shown to be a potent molecule in stimulating adhesion protein expression in human endothelial cells (Fig. 2). The finding that S1P up-regulated adhesion molecules not only in cell-surface expression but also in mRNA levels and activation of NF-κB, an essential transcription factor for these proteins, suggests a potential role of S1P linking signal transduction to gene expression. The strong signaling role of S1P has been reported in mediating a variety of cellular functions such as calcium mobilization (25, 26), cell motility, and proliferation (27–29). S1P is believed to serve both as an intracellular second messenger (10, 15) and as an extracellular ligand for the G-protein-coupled receptor Edg-1 (30, 31). These two conceptually different signaling roles have been claimed for this single lipid molecule in a cell type- and phenomenon-specific manner, making S1P a unique bioactive signal molecule (29). At this stage the site of S1P action in the induction of adhesion protein expression is not known. However, two sets of observations suggest that S1P acts at least in part intracellularly. (*i*) The effective concentrations of S1P were on the order of micromolar ($EC_{50} \approx 1$ μM), which is in contrast with the nanomolar amounts needed for effects of S1P through Edg-1 (30). Interestingly, it has been previously noted that the effective dose of S1P required for proliferation was micromolar, whereas the dose for cell motility regulation was nanomolar (29). (*ii*) Neither pertussis toxin (a G protein inhibitor) nor suramin (a nonspecific inhibitor of phospholipid receptors; ref. 32) caused a >30% of inhibition on the effects of S1P in the induction of adhesion molecules (data not shown).

In this report we demonstrated that S1P is a mediator in TNFα-induced endothelial cell activation. (*i*) TNFα induced a rapid and transient activation of SKase, with corresponding increases in S1P levels in HUVEC. (*ii*) The addition of S1P mimicked TNFα effect on the induction of adhesion proteins. (*iii*) The blockage of S1P production by DMS, a competitive inhibitor of SKase, significantly decreased TNFα-induced adhesion protein expression. In contrast, DMS did not inhibit the expression of adhesion molecules induced by S1P (Fig. 2*B*) or endotoxin (lipopolysaccharide, data not shown), indicating a specific inhibition on TNFα-dependent SKase activation. (*iv*) The inhibitory effect of DMS was reversed by addition of exogenous S1P. It is thus likely that SKase activation and S1P generation are involved in the TNFα-promoted endothelial cell activation.

In an attempt to define the intracellular targets through which S1P modulates TNFα signaling, we examined the activation of MEK/ERK and NF-κB. Previous studies have established that NF-κB is essential for regulating adhesion protein expression (21). Mitogen-activated protein kinases containing multiple subgroups such as ERK, JNK, and p38 kinases were also shown to be involved in endothelial cell gene expression in responses to TNFα stimulation (17, 20). Our preliminary data (not shown) show that the inhibition of ERK by a MEK-specific inhibitor (PD098059) significantly reduces TNFα-induced adhesion molecule expression, in agreement with previous reports showing the inhibition of TNFα-dependent gene expression by this inhibitor

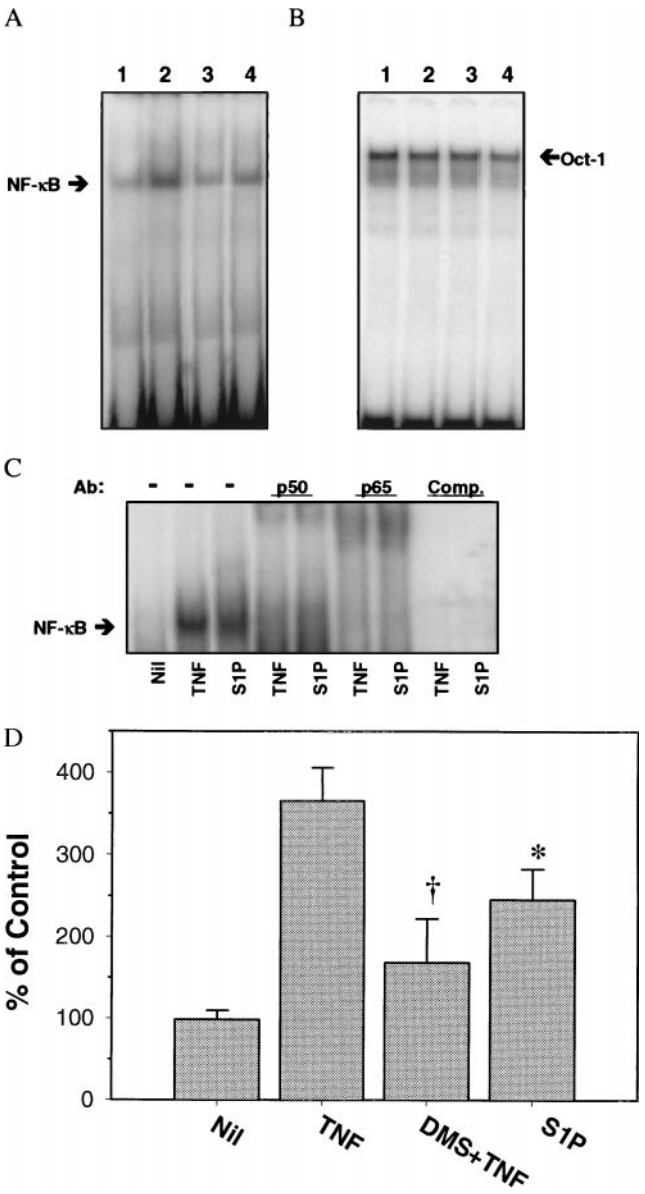

FIG. 5. Effect of SKase activity on NF-κB activation. NF-κB (*A*) and Oct-1 (*B*) binding activity were measured by electrophoretic mobility-shift assay after 30-min treatment with a vehicle (lane 1), TNFα (TNF, 100 units/ml, lane 2), DMS (5 μM) plus TNFα (lane 3), and S1P (5 μM, lane 4), respectively. (*C*) The specific NF-κB binding complexes were identified by the supershift gel assay with anti-p50 and anti-p65 antibodies and by competition analyses with the addition of a 50-fold molar excess of unlabeled NF-κB oligonucleotides. (*D*) Bar graph depicts relative binding activity of NF-κB quantified by the PhosphorImager from *A*. Values represent mean ± SD in percent of control from three independent experiments. *, $P < 0.01$, compared with control; †, $P < 0.01$, vs. TNFα stimulation.

(33, 34). We found that S1P mimicked the effect of TNFα in stimulating ERK but not JNK activation, and again DMS markedly reduced TNFα effect on ERK and NF-κB signaling cascades (Figs. 4 and 5). By contrast, neither ceramide nor sphingosine could activate these signals and induce adhesion molecule expression. Taken together, our data demonstrated an important role of SKase pathway in mediating TNFα stimulation on endothelial cells. It is noted that SKase activation may not be the only pathway to mediate endothelial cell activation by TNFα, as the blockage of TNFα effects by a saturating dose of DMS was not complete, and stimulation of ERK and NF-κB can certainly be achieved by other pathways (20, 21). Further understanding of the precise role of the SKase pathway in regulating endothelial cellular functions is expected, as this critical enzyme has just been cloned (35).

In conclusion, we have demonstrated that TNFα, a pleiotropic cytokine, induces activation of SKase and generation of S1P, which in turn may serve as a second messenger to mediate TNFα-induced endothelial cell activation and adhesion molecule expression. This could provide a mechanism for the regulation of endothelial activation during the systemic inflammatory and immune responses.

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